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14. ABSTRACT

This project focuses on the novel finding from our group that the serine-threonine kinase Akt1 partitions into specialized membrane microdomains, termed lipid rafts, and that this localization event strongly influences the nature of Akt1 signaling. Lipid rafts are cholesterol-enriched membrane microdomains that serve as signal transduction platforms by sequestering and excluding signaling proteins and by harboring multi-protein complexes. Evidence was presented in the original proposal that in prostate cancer cells critical cell survival cues are processed via lipid rafts, which are dependent on cholesterol for signal transduction. This is a significant finding because the Akt1 kinase is a central signaling protein that is frequently activated in prostate cancer. I have hypothesized in this project that cholesterol accumulation in prostate cancer cells may promote oncogenesis by altering the nature of Akt1 signals that flow through lipid raft microdomains. The purpose of this project is to identify the mechanism of Akt1 recruitment to cholesterol-rich microdomains and to explore the biological consequences for regulation of this important kinase. Several new lines of evidence consistent with my hypothesis have been produced in the second year and are described and summarized in this report.

15. SUBJECT TERMS
Prostate cancer, Akt1, cholesterol, lipid raft, kinase, phosphorylation

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Progress report for 02/14/2008 - 07/14/2009

INTRODUCTION

This project focuses on the novel finding from our group that the serine-threonine kinase Akt1 partitions into specialized membrane microdomains, termed lipid rafts, and that this localization event strongly influences the nature of Akt1 signaling. Lipid rafts are cholesterol- and sphingolipid-enriched membrane microdomains that serve as signal transduction platforms by sequestering and excluding signaling proteins and by harboring pre-formed multi-protein complexes. Evidence was presented in the original proposal that in prostate cancer cells critical cell survival cues are processed via lipid rafts, which are dependent on cholesterol for signal transduction. This is a significant finding because the Akt1 kinase is a central signaling protein that is frequently activated in prostate cancer. I have hypothesized in this project that cholesterol accumulation in prostate cancer cells may promote oncogenesis by altering the nature of Akt1 signals that flow through lipid raft microdomains. The purpose of this project was to identify the mechanism of Akt1 recruitment to cholesterol-rich microdomains and to explore the biological consequences for regulation of this important kinase.

BODY

In year 2, I have completed Tasks 1 and 2 and made significant progress on Task 3 and 4.

Task 1 and 2: Determine the unique functional consequences of Akt recruitment to lipid rafts for signaling processes in prostate cancer cells.

As described in the Annual Summary Report for the period 15 Feb 2007- 14 Feb 2008, I have now completed Task 1. i)- iii) and demonstrated the construction and expression of raft-targeted as well as raft-excluded Akt molecules (Fig. 1) These various constructs were then tested regarding their lipid raft distribution as proposed in Task 2 i).

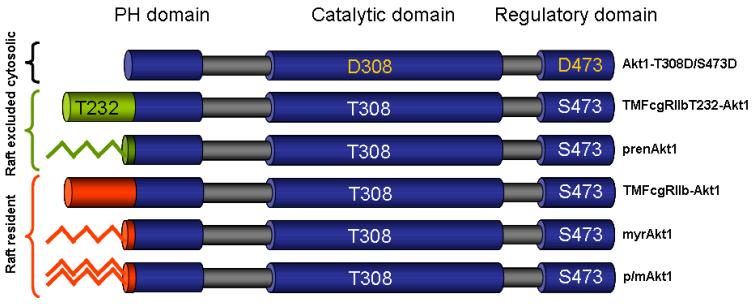

Figure 1: Overview of differentially targeted Akt1 fusion proteins. The Akt1 kinase features three characteristic domains including the pleckstrin homology (PH) domain, the catalytic domain with the Thr308 phosphorylation site and the regulatory domain with its Ser473 phosphorylation site. The PH domain of Akt1 is fused N-terminally to various targeting sequences that will guide Akt1 to rafts (red) or exclude it from this membrane compartment (green).

In summary, comparison of cytoplasmic/non-raft membrane fractions and raft-fractions after differential extraction revealed that partitioning of Akt1 into the lipid raft compartment is not influenced by mutating the two major regulatory sites threonine 308 and serine 473. The phosphomimetic double mutant Akt-T308D/S473D (uppermost construct, Fig. 1) did not show a lipid raft distribution that was different from the wild type and proved to be well suited as an activated control construct. Interestingly, the Akt-S473D phosphomimetic mutant, which was not included in the original proposal (not shown in Fig. 1), displayed altered distribution with increased accumulation of the kinase in the lipid raft compartment. Moreover, the kinase-dead mutant of Akt1 (K179M), which is unable to bind ATP in the active site, accumulated predominantly in lipid rafts (for details see Annual Summary Report March 2008). Consequently, I was able to identify an important function for serine 473 and lysine 179 in regulating the admission of Akt1 to the lipid raft signaling platform. Raft localization was also confirmed with construct TMFcγRIIb-Akt1, which was clearly enriched in raft-membranes (fourth construct from the top, Fig.1). Introduction of a single amino-acid substitution in the transmembrane domain of TMFcγRIIb-Akt1 dramatically altered raft localization and resulted in virtually complete exclusion of Akt1 from the lipid raft compartment (second construct from the top, Fig. 1). Unfortunately, the proposed alternative approach to create a raft-excluded Akt1 variant (third construct from the top, Fig. 1), proved to be incompatible with Akt1 activity. The prenylation signal derived from K-Ras rendered Akt1 catalytically inactive when fused to the N-terminal PH-domain (Fig. 1). Fusion of this motif to the Akt C-terminus resulted in very low expression levels. Despite this setback, I was able to generate a complete set of constructs to study the consequences of raft-targeting and raft-exclusion of the Akt1 kinase for cancer cell proliferation and cell survival.

Having demonstrated expression and verified subcellular localization, I was able to proceed to Task 2 ii) and address the question of how the raft-resident population of Akt1 affects cellular behaviors. I focused in particular on the sensitivity of raft-resident Akt1 to lipid raft disruption using the cholesterol binding drug methyl-β-cyclodextrin (CD). To confirm the cholesterol sensitivity of lipid raft-resident Akt1, LNCaP cells were treated with CD and fractionated into cytosol/ nonraft membranes (C+M) and raft membranes. Akt1 was immunoprecipitated from each fraction and blotted with antibodies to total and phospho-S473 Akt1. Cyclodextrin treatment did not appreciably alter the amount or extent of phosphorylation of Akt isolated from the C+M fraction. In contrast, CD ablated phosphorylation of raft-resident Akt1.

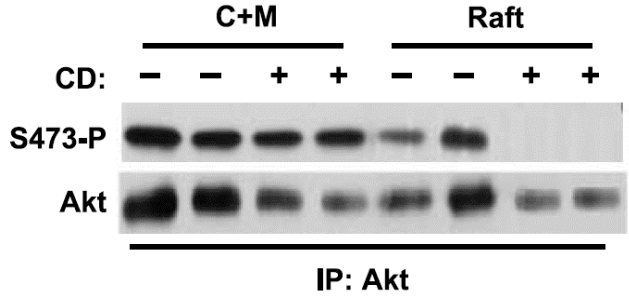

Figure 2: A population of endogenous Akt1 resides in a cholesterol-rich membrane fraction. Akt1 was immunoprecipitated from LNCaP cells exposed to cyclodextrin (CD) and fractionated into C+M and raft fractions. Immunoprecipitated eluates were blotted with antibodies to total and phospho-Akt1.

To further understand how signals transmitted from raft-resident Akt1 differ from Akt signaling at other membrane locations, I used stably transfected LNCaP prostate cancer cells expressing a raft-targeted form of Akt1 (for a detailed description regarding the generation of this cell line see Annual Summary Report March 2008). The LNCaP pros-

tate cancer cells expressing this raft-resident MyrAkt1 (second construct from the bottom, Fig. 1) were then tested regarding their cholesterol sensitivity by manipulating membrane cholesterol levels with either cyclodextrin alone, water-soluble cholesterol alone or cyclodextrin treatment followed by restoring membrane cholesterol. As shown in Figure 3, CD-treatment led to a decrease in membrane cholesterol compared to untreated cells and essentially a complete loss of MyrAkt1 from rafts.

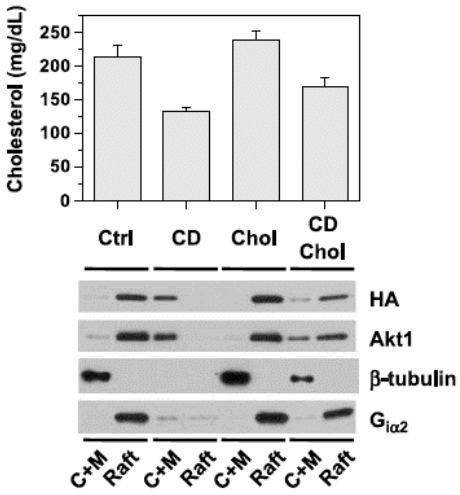

Figure 3: Oncogenic Akt1 is enriched in lipid rafts and sensitive to cyclodextrin. Stably transfected LNCaP cells expressing MyrAkt1 were treated with either cyclodextrin (CD) or water-soluble cholesterol (Chol) or with CD followed by cholesterol treatment (CD Chol). Cells incubated in serum-free medium served as controls. Following differential extraction of Triton-soluble cytoplasmic/non-raft membrane fractions and octylglucoside-soluble lipid raft fractions, proteins were blotted and detected with antibodies to Akt1, the HA-tag, tubulin and G-protein subunit $\alpha 2$ as the raft marker.

Cholesterol repletion restored membrane cholesterol levels to ~80% of the basal level and reestablished the basal distribution of MyrAkt1. Cholesterol treatment in the absence of depletion increased membrane cholesterol by ~12% and led to a modest but detectable increase of MyrAkt1 in rafts.

After having established that the raft-resident population is especially sensitive to lipid raft disruption using CD, I further elucidated whether this sensitivity is reflected in the susceptibility to apoptotic triggers such as the PI3-kinase inhibitor LY294002, which I had proposed in Task 2 ii). This reflection was based on a previous finding from our group that demonstrated the induction of apoptosis in LNCaP cells after treatment with LY294002. Therefore, the stably transformed LNCaP/MyrAkt1 and control cells expressing LacZ were exposed to the PI3K inhibitor LY294002 for 24hours and its apoptotic effect on LNCaP cells was assessed by flow cytometry (Fig. 4). Surprisingly, LNCaP/MyrAkt cells were almost completely insensitive to PI3K inhibition, in contrast to LNCaP/LacZ cells that displayed significant induction of apoptosis (inset). However, the cytoprotective effect of MyrAkt1 was diminished by depletion of membrane cholesterol prior to treatment with LY294002, suggesting that anti-apoptotic signals are transmitted, at least in part, by the raft-resident population of Akt1.

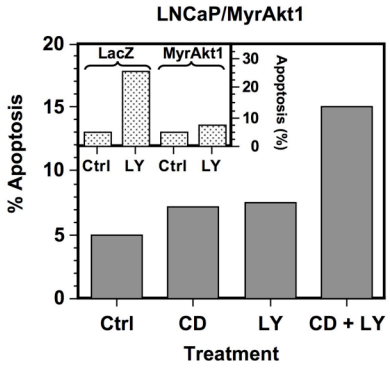

Figure 4: Activated Akt1 confers resistance to apoptosis induced by PI3K inhibition. LNCaP cells stably expressing MyrAkt1 or control cells expressing LacZ (inset) were treated without or with LY294002 for 24 hours and the extent of apoptosis determined by flow cytometry. LNCaP/MyrAkt1 cells were treated without (Ctrl) or with 5mM cyclodextrin (CD) for 1 hour, 10uM LY294002 (LY) for 24 hours or both agents (CD + LY) and harvested for flow cytometry. Data are presented as apoptotic cells (sub-G1-peak) expressed as a percentage of the total cell population and are representative of two independent trials.

In a second set of experiments, the raft-resident population of Akt1 was further examined in regards to its sensitivity to specific inhibitory proteins, as proposed in Task 2 iii). Based on my hypothesis that Akt1 interacts with a specific set of regulatory proteins that are admitted to the lipid raft compartment, I decided to focus explicitly on inhibitory proteins that also show enrichment in rafts. Interestingly, our laboratory has begun to specifically explore the lipid raft microdomain in search for Akt1 interacting proteins that transit raft membranes. Immunoprecipitation of Akt1 from lipid raft fractions of LNCaP cells and subsequent analysis of co-precipitating proteins by MALDI-TOF mass spectrometry identified the mammalian serine/threonine sterile 20 (STE20)-like kinase Mst1 as a potential Akt1 interacting protein. Mst1 was detected primarily in Akt1 complexes isolated from raft fractions, which suggests that complex formation between endogenous Mst1 and Akt1, as measured by co-IP, occurs preferentially in lipid raft membrane fractions (Fig. 5. left). Moreover, our laboratory recently reported that expression of Mst1 inhibits endogenous Akt1 activity in LNCaP cells and induces apoptosis, which is in agreement with the requirement for constitutive signaling through the PI3K/Akt pathway for survival in this cell line. Consequently, I decided to test the sensitivity of raft-resident Akt1 to the inhibitory activity of Mst1. As expected, PI3K inhibiton by the LY compound induced apoptosis in LNCaP cells (Fig. 5, right). Surprisingly, silencing of Mst1 expression by siRNA antagonized the apoptotic effect of LY, implicating Mst1 in the apoptotic mechanism induced by PI3K inhibition.

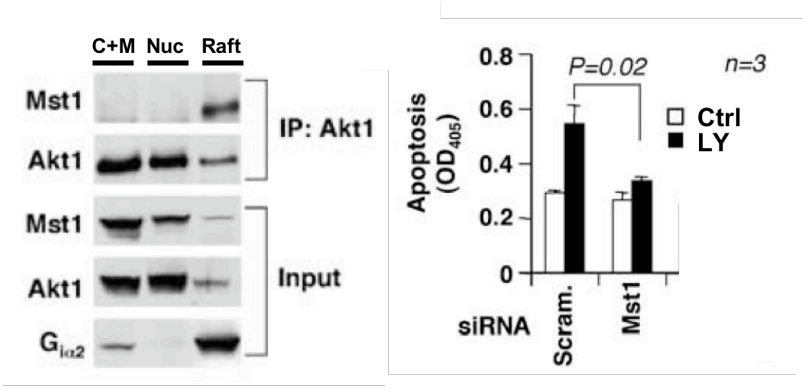

Figure 5: Mst1 and Akt1 interact preferentially in lipid raft fractions. Immunoprecipitated Akt1 from cytosol/non-raft membranes, nuclear or raft fractions displays complex formation with Mst1 only in the lipid raft domain (left). Induction of apopotosis in LNCaP cells by treatment with LY involves Mst1 and is diminished in absence of this kinase.

Task 3 and 4. Determine the functional role of HSP90 family members in Akt1 activity and recruitment to lipid rafts in prostate cancer cells.

As described in the Annual Summary Report for the period 15 Feb 2007- 14 Feb 2008, I encountered difficulties with retrieving the correct HSP89αΔN cDNA from three different prostate cancer cells. Therefore, decided to clone this gene with a nested primer strategy. Even though I was able to accomplish construction and cloning of the HSP89αΔN gene (see Annual Summary Report March 2008 for details), I was not able to achieve expression of this construct in prostate cancer cells.

In consultation with Professor Freeman, I decided to embark on a different stategy to elucidate recruitment of the Akt1 kinase to lipid rafts in prostate cancer cells. As mentioned above, our laboratory has begun to specifically explore the lipid raft microdomain in search for Akt1 interacting proteins that transit raft membranes. Based on this systematic search, we identified the heterogeneous nuclear ribonucleoprotein K (hnRNPK) as a Akt1 binding protein in lipid rafts. hnRNPK acts as a scaffold protein that integrates signaling cascades by facilitating the cross talk between kinases. Therefore, we hy-

pothesized that, similar to HSP90, hnRNPK could serve as a scaffold protein for Akt1 and may facilitate recruitment of Akt1 to the lipid raft compartment. Currently, we are trying to identify, which domain of hnRNPK is responsible for complex formation with Akt1, an experiment that was originally proposed for HSP90 in Task 4. iii). To this end, I generated various truncated versions of hnRNPK and tested them in *in-vitro* GST binding studies (Fig. 6).

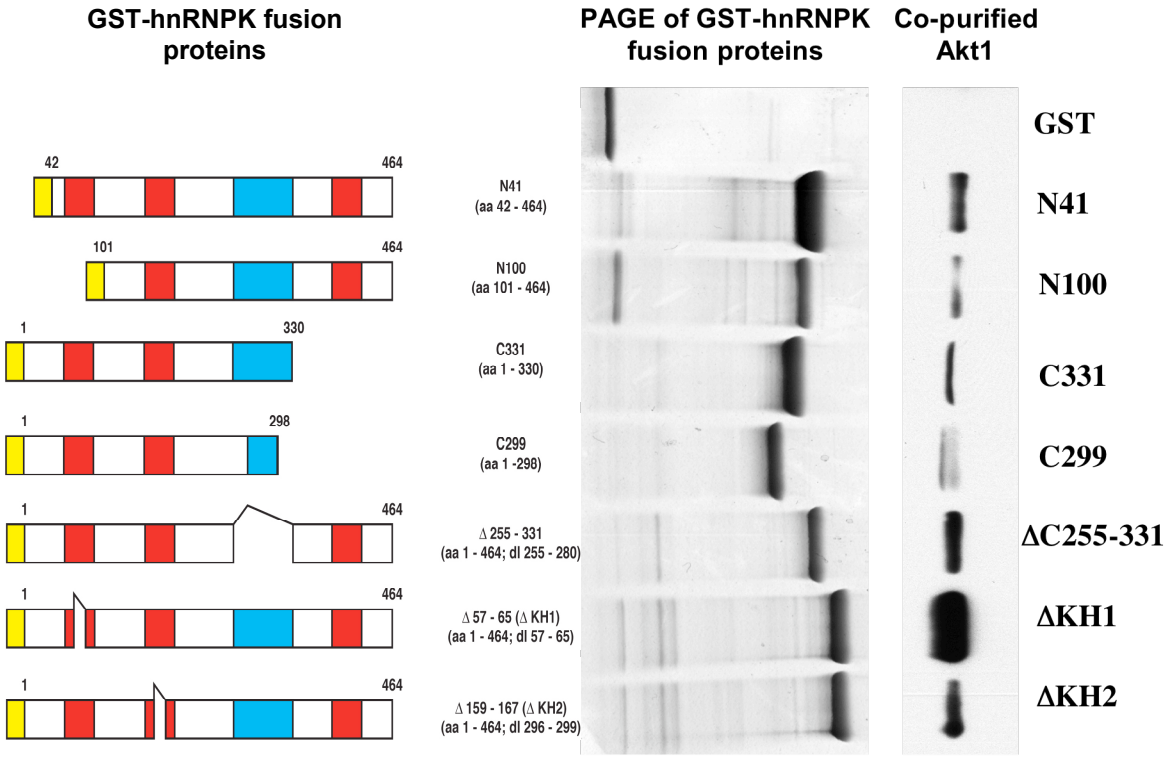

Figure 6: Domain structure of hnRNPK and complex formation with Akt1. An overview of the hnRNPK truncation mutants used in the binding assay as GST fusion proteins (left). Purification of GST-hnRNPK fusion proteins by affinity chromatography reveals co-purification of Akt1 (right).

The hnRNPK protein binds with high affinity to Akt1 as demonstrated by purification of GST-hnRNPK fusion proteins with subsequent western-blotting and detection of Akt1 with an Akt1-specific 2H10 antibody. Interestingly, deletion of the KH1 domain facilitates binding of Akt1 to hnRNPK, whereas the C-terminus seems to be important for Akt1 binding. These studies will help us to understand, which domains of hnRNPK are important for complex formation with Akt1 and whether hnRNPK can function as a scaffold protein that is instrumental in Akt1 kinase membrane recruitment.

KEY RESEARCH ACCOMPLISHMENTS

- Raft-resident Akt1 was identified as an important signaling molecule that confers a cytoprotective effect.
- I have shown that Akt1 activity is specifically regulated in the raft compartment, which facilitates interaction of complex formation of Akt1 and its inhibitory kinase Mst1

- Myristoylated Akt1, which is an oncogene, is over-represented in lipid raft fractions in comparison to wild type Akt1 and mutations at important regulatory sites of Akt1 alter its lipid raft distribution.
- This is the first evidence that cholesterol is a direct regulator of Akt-dependent signaling in prostate cancer cells.

CONCLUSION

In summary, I have obtained evidence that signals emanating from raft-resident Akt1 provide important cell survival cues. These signals are sensitive to cholesterol depletion suggesting that anti-apoptotic signals derive from Akt1 when localized to rafts. Consequently, the findings of my research as a Department of Defense Prostate Cancer Research Program Scholar suggest a direct mechanistic link between cholesterol and cell survival signaling in tumor cells and may be functionally relevant to the reported chemopreventive benefit of long-term use of cholesterol-lowering drugs in certain cancers.

REPORTABLE OUTCOMES

- Nishit Mukhopadhyay, **Martin H. Hager**, Jayoung Kim, Delia Lopez, Dolores Di Vizio, Bekir Cinar, Rosalyn Adam and Michael R. Freeman (2009) Scaffold Attachment Factor B1 is a direct target of nuclear Akt and mediates growth arrest and repression of androgen receptor transcriptional activity in human prostate cancer cells. (submitted).

- Dolores Di Vizio, Jayoung Kim, **Martin H. Hager**, Matteo Morello, Marc A. Rubin, Rosalyn M. Adam, Wei Yang and Michael R. Freeman (2009) Oncosome Formation in prostate cancer: Association with a region of frequent chromosomal deletion in metastatic disease. *Cancer Res.* 69: (13) 5601- 5609.

- Nishit Mukhopadhyay, Jayoung Kim, Bekir Cinar, Aruna Ramachandran, **Martin H. Hager**, Rosalyn M. Adam, Pradip Raychaudhuri, Arrigo DeBenedetti and Michael R. Freeman (2009) Heterogeneous Nuclear Ribonucleoprotein K is a Novel Regulator of Androgen Receptor Translation. *Cancer Res.* 69: (6): 2210-2218.